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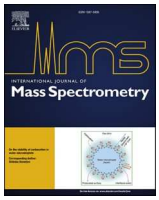

Absolute quantitation of peptides and proteins by coulometric mass spectrometry after derivatization

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ABSTRACT

Peptide/protein quantitation using mass spectrometry (MS) is advantageous due to its high sensitivity. Traditional absolute peptide quantitation methods rely on making calibration curves using peptide standards or isotope-labelled peptide standards, which are expensive and take time to synthesize. A method which can eliminate the need for using standards would be beneficial. Recently, we developed coulometric mass spectrometry (CMS) which can be used to quantify peptides that are oxidizable (e.g., those containing tyrosine or tryptophan), without using peptide standard. The method is based on electrochemical oxidation of peptides followed by MS measurement of the oxidation yield. However, it cannot be directly used to quantify peptides without oxidizable residues. To extend this method for quantifying peptides/proteins in general, in this study, we adopted a derivatization strategy, in which a target peptide is first tagged with an electroactive reagent such as monocarboxymethylene blue NHS ester (MCMB-NHS ester), followed with quantitation by CMS. To illustrate the power of this method, we have analyzed peptides MG and RPPGFSPFR. The quantification error was less than 5%. Using RPPGFSPFR as an example, the quantitation sensitivity of the technique was found to be 0.25 pmol. Furthermore, we also used the strategy to quantify proteins cytochrome C and β-casein with an error of 2–26 %.

1. Introduction

Monitoring protein expression level is important for studying various diseases [1]. In discovery-based proteomics, a broad analysis of the proteome is carried out to look for quantitative differences in proteins, using relative quantitation [2–5]. Relative quantitation methods provide information regarding the protein abundance changes during different conditions, but do not provide a specific concentration. However, in many instances such as biomarker-based diagnostic tests, absolute quantitation providing the actual concentration of a specific peptide or a protein in a sample is highly preferred. Historically, UV has been used to measure the protein concentration. However, this technique depends on the presence of tryptophan and tyrosine as these amino acids show strong absorbance at 280 nm. If the primary sequence of the protein shows few tryptophans or tyrosines, it shows erroneous results [6]. Besides, the Bicinchoninic assay (BCA) is used for routine analysis of protein quantitation, however, this method is prone to interference with reducing agents [7]. The Folin-Lowry assay is a calorimetric test which is easy to perform but also suffers interference from commonly used chemicals such as EDTA, Tris, carbohydrates, and reducing agents [8]. High resolution 2D gel electrophoresis with the Bradford assay is also used for protein quantitation [9]. The issue is that the technique is labor intensive and the quantitation sensitivity is poor with typical limit of detection in the μg/mL range [10] whereas, with the mass spectrometry technique, typical limits of detection are in the ng/mL range [11]. A mass spectrometric method for absolute quantification of proteins is highly desirable as the method provides a specific concentration with high sensitivity and specificity.

Quantitation of peptides and proteins by MS can be carried out either by the isotope labeling strategy or the label free strategy [12–17]. In isotope llabeling strategy for relative quantitation, a stable isotope-labelled peptide that is chemically identical to its native counterpart is used and quantification is achieved by comparing their ion signal intensities between the isotope labelled standard and the native counterpart [18]. Various methods of isotope labelling are available. Some of the most popular methods include tandem mass tags (TMT) [19], isotope-coded affinity tags (ICAT) [20], stable isotope labeling by amino acids in cell culture (SILAC) [21], isobaric tags for relative and absolute quantitation (iTRAQ) [22], metal element chelated tags (MECT) [23] and isotope-coded protein labeling (ICPL) [24]. With

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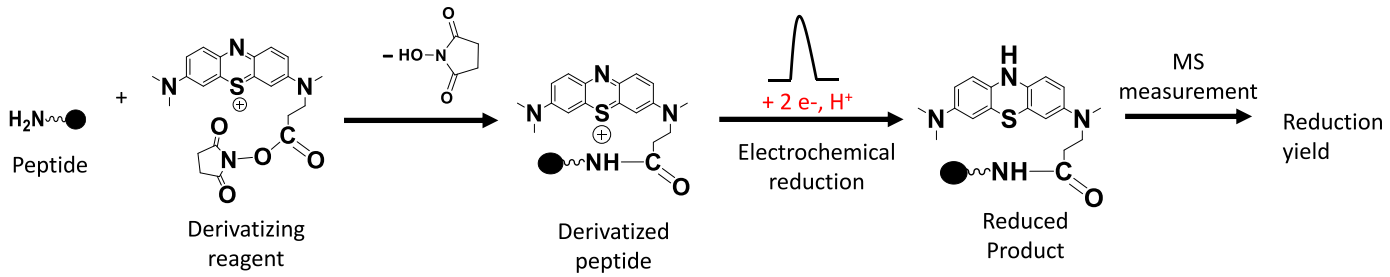

Scheme 1. Our approach for absolute quantitation of a peptide by CMS after derivatization of its amino group (z = 2 for reducing methylene blue tag).

label-free methods for relative quantitation, protein quantification is generally based on ion intensity changes between the standard and native proteins which are run as separate samples [3]. In absolute quantitation, a calibration curve is prepared using peptide standards, or isotope-labelled standards [25,26]. The three most popular isotope labeling methods for absolute quantitation are absolute quantification (AQUA), quantification conCATamer (QconCAT) and protein standard absolute quantification (PSAQ). In an AQUA experiment, synthetic AQUA peptides are added to the sample right before LC-MS analysis, serving as standards [27]. QconCATs are proteins encoded by synthetic genes that are concatamers of peptide internal standards. The native protein and the QconCAT protein are digested by trypsin and digested peptides are compared by mass spectrometry for quantification [28]. The protein digestion efficiency issue of AQUA method is thus rectified with QconCAT to some extent [26]. In PSAQ methodology, DNA sequence to code for PSAQ protein is cloned into a plasmid and expressed in an expression medium such as *E. coli* to produce standard proteins for quantitation [29]. Nevertheless, although these absolute quantitation methods are successful, they need standards, whose syntheses can be expensive, complicated and time-consuming. It would be ideal to have a standard-free absolute quantitation method available to quantify peptides and proteins for proteomics research.

Recently, we developed coulometric mass spectrometry (CMS) [30–37] for absolute quantitation of electroactive analytes using liquid chromatography/electrochemistry/mass spectrometry (LC/EC/MS) apparatus. The method is based on electrochemical oxidation/reduction of analytes followed by MS to measure the oxidation yield. Electrochemical reaction results in an electric current response, which can be integrated over time to calculate the electric charge Q involved in the redox reaction. According to Faraday's Law, Q is proportional to quantity of the oxidized/reduced analyte: Q = nzF, where n is the moles of the oxidized/reduced analyte, z is the number of electrons transferred per molecule during the redox reaction, and F is the Faraday's constant (9.65×10^4 C/mol). Therefore, the moles of the oxidized/reduced analyte can be calculated as n = Q/zF. Meanwhile, upon oxidation or reduction, the target analyte shows a reduced intensity in the acquired MS spectra, and the relative analyte ion intensity change, Δi, reflects the redox conversion yield. Thus, the moles of the oxidized/reduced analyte, in combination with the conversion yield, can be used to calculate the total amount of the analyte. In other words,

Total amount of the analyte

=(amount of the oxidized/reduced analyte)/(the conversion yield)

=(Q/zF)/Δi

$$= Q/(zF\Delta i) \quad (1)$$

Using this CMS technique, a wide variety of molecules were quantified. Small molecules with electroactive functional groups such as dopamine and norepinephrine as well as drug impurity of nitrosamines were successfully quantified [30–32]. We have also shown that electroactive peptides such as those containing amino acid cysteine, tyrosine and tryptophan can be accurately quantified by CMS [33,35,36]. Proteins can be digested and the surrogate peptides containing electroactive amino acids are measured to obtain the protein quantities [33]. However, the method is limited to electroactive species and cannot be directly used to quantify peptides without oxidizable peptides.

To tackle this problem and make CMS more generally applicable for peptide/protein quantitation, in this study, we adopted a strategy of derivatizing peptides with an electroactive reagent of MCMB-NHS ester (illustrated in Scheme 1). Similar to the derivatization in the fluorescence detection strategy where molecules are derivatized with fluorescent tags for improving the selectivity and sensitivity, peptides are derivatized with electrochemical tags in our strategy (note that, unlike fluorescence-based quantitation, our method does not need calibration curve or standards for quantitation) [41–44]. By this method, the peptide which does not contain an electroactive amino acid can be converted into one carrying a methylene blue moiety, a well-known electrochemical tag and thus can be quantified by CMS. We applied this modified CMS method for absolute quantitation of peptides and proteins. Our results show that the derivatization strategy successfully extended the scope of our CMS method applications in quantitation.

2. Experimental section

2.1. Chemicals

MG (Met-Gly), cytochrome C, β-casein and trypsin were purchased from Sigma-Aldrich (Saint Louis, MO). RPPGFSPFR (Bradykinin) was purchased from Genscript (Piscataway, NJ). MCMB-NHS ester was purchased from Emp-Biotech (Howell, NJ). Formic acid and acetonitrile were obtained from Fisher Chemical (Fair Lawn, NJ), and deionized water used for sample preparation was obtained using a Millipore purification system (Burlington, MA).

2.2. Proteolytic digestion

We prepared 100 μL of 100 μM cytochrome C from *Saccharomyces cerevisiae* using water (pH was adjusted to 8 by NaOH) followed by adding 5 μL of 1 μg/μL trypsin solution. The protein to trypsin ratio was 25:1 by weight. The protein sample was then incubated at 37 °C overnight. The digested protein was derivatized with MCMB-NHS ester for 1.5 h. The concentration ratio of the MCMB-NHS ester: cytochrome C in the reaction sample was 1:1. After derivatization reaction, the sample was quenched with hydroxylamine to remove the remaining MCMB-NHS and further diluted to a 1.19 μM final concentration with water/acetonitrile/formic acid (85: 15: 0.1 by volume) for CMS quantification.

We prepared 100 μL of 100 μM β-casein from equine heart using water (pH was adjusted to 8 by NaOH) followed by adding 10 μL of 1 μg/μL trypsin solution. The protein to trypsin ratio was 25:1 by weight. The protein sample was incubated at 37 °C overnight. The digested protein was derivatized with MCMB-NHS ester for 12 h. The concentration ratio of the MCMB-NHS ester: β-casein in the reaction sample was 8:1. After derivatization reaction, the sample was diluted to a 5.05 μM final concentration with 50: 50: 0.1 water/acetonitrile/formic acid for CMS quantification. The 0.1 % formic acid in the above dilution solution

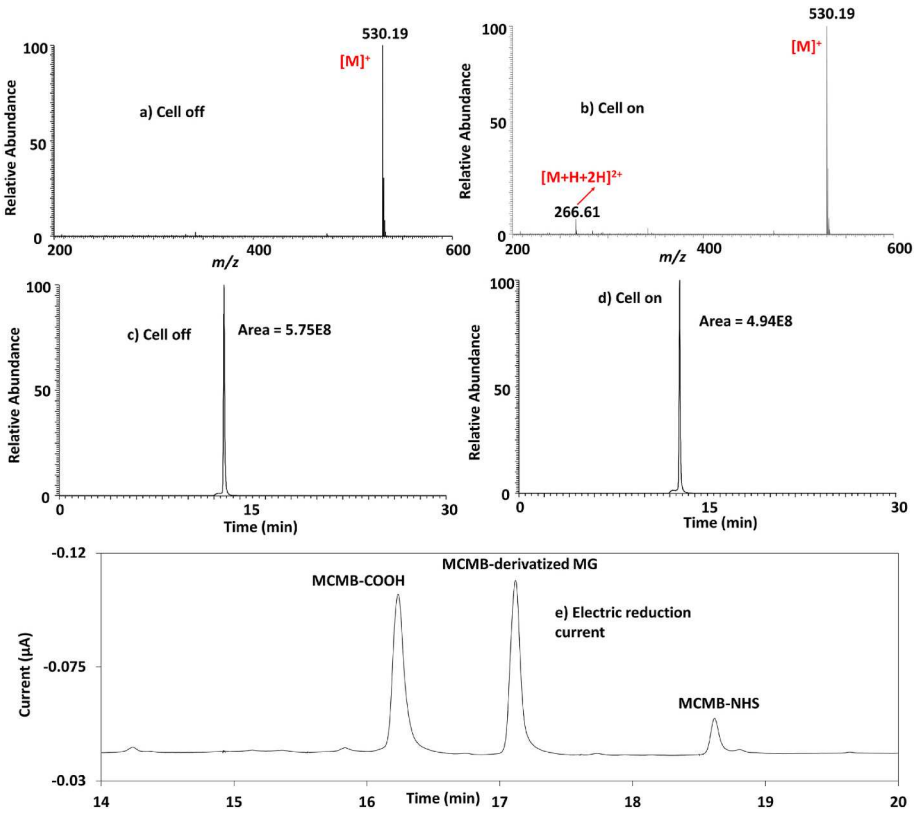

Fig. 1. Mass spectra of MCMB-derivatized MG a) before electrochemical reduction and b) after electrochemical reduction; EIC peak of MCMB-derivatized MG c) before reduction and d) after reduction; e) Diagram showing the electrochemical reduction currents.

decreased the pH and thus quenched the solution to prevent further reaction of protein digest with MCMB-NHS ester during CMS analysis.

2.3. Instrumentation

For the CMS experimental setup using LC/EC/MS apparatus (illustrated in Scheme S1, Supporting Information), a Waters ultra-performance liquid chromatography setup (UPLC, Milford, MA) was coupled with a BASi electrochemical flow cell (West Lafayette, IN). The BASi electrochemical cell was equipped with a 3 or 6-mm *i. d.* glassy carbon working electrode (WE) and an Ag/AgCl reference electrode (RE). A BEH C18 reversed phase column (2.1 mm × 50 mm, 1.7 μm) was installed for the UPLC separation. A negative potential of −0.2 V vs. Ag/AgCl was applied to the WE electrode for reduction of LC-separated peptides tagged with MCMB-NHS ester. To measure the reduction yield of a derivatized peptide, a control sample (or the "cell off" sample as mentioned below) was also injected for the analysis under the same LC/EC/MS conditions except that the cell potential was not applied. The redox current response was monitored and recorded by a potentiostat and integrated by software OriginPro 2019 to calculate the total electric charge Q involved in the reduction reaction. The eluate flowing out of the cell was subsequently analyzed using online electrospray ionization mass spectrometry (ESI-MS). MS data were collected using a high-resolution Q-Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA). The sheath gas flow rate was 10 L/h. The spray voltage was +4 kV and the capillary temperature was kept at 250 °C. MS, MS/MS spectra and extracted ion chromatograms (EICs) of peptides were acquired by Thermo Xcalibur (4.1).

For the proof-of-concept experiment, we first derivatized peptide MG with MCMB-NHS ester. The mobile phase flow rate was set at 0.3 mL/min. A gradient elution program (mobile phase A: water with 0.1 % formic acid and mobile phase B: acetonitrile with 0.1 % formic acid) starting with 95 % A for 1 min, 95 % A to 65 % A in 24 min, 65 % A to 10 % A in 1 min, linear gradient at 10 % A for 3 min and then 10 % A to 95 % A in 0.1 min. The derivatized peptide concentration used was 1.50 μM and the injection volume was 10 μL. Also, we derivatized RPPGFSPFR with MCMB-NHS ester. The mobile phase flow rate was set at 0.1 mL/min. A gradient elution program (mobile phase A: water with 0.1 % formic acid and mobile phase B: acetonitrile with 0.1 % formic acid) starting with 95 % A for 1 min, 95 % A to 64 % A in 25 min, 64 % A to 30 % A in 1 min, linear gradient at 30 % A for 2 min and then 30 % A to 95 % A in 0.1 min. The derivatized peptide concentration used for the analysis was 3.75 μM and the injection volume was 3 μL.

In addition, to demonstrate the quantification of proteins using this technique, two proteins cytochrome C, and β-casein were chosen for CMS quantitation test. After digestion and derivatization, a 10 μL cytochrome *c* protein digest that was derivatized with MCMB-NHS ester as described above (protein concentration: 1.19 μM) was injected for LC separation. The mobile phase flow rate was set at 0.3 mL/min. A gradient elution program (mobile phase A: water with 0.1 % formic acid and mobile phase B: acetonitrile with 0.1 % formic acid) starting with 95 % A for 1 min, 95 % A to 81.2 % A in 7 min, 81.2 % A to 78.6 % A in 24 min, 78.6 % A to 10 % A in 1 min, linear gradient at 10 % A for 3 min and then 10 % A to 95 % A in 0.1 min. For β-casein protein digest sample, the protein was digested and derivatized as described above. A 5 μL β-casein protein digest that was derivatized with MCMB-NHS ester (protein concentration: 5.05 μM) was injected for LC separation. The mobile phase flow rate was set at 0.1 mL/min. A gradient elution program (mobile phase A: water with 0.1 % formic acid and mobile phase B: acetonitrile with 0.1 % formic acid) starting with 95 % A for 1 min, 95 % A to 60 % A in 34 min, 60 % A to 20 % A in 1 min, linear gradient at 20 % A for 3 min and then 20 % A to 95 % A in 0.1 min.

3. Results and discussion

The combination of MS and electrochemistry (EC) has been a topic of

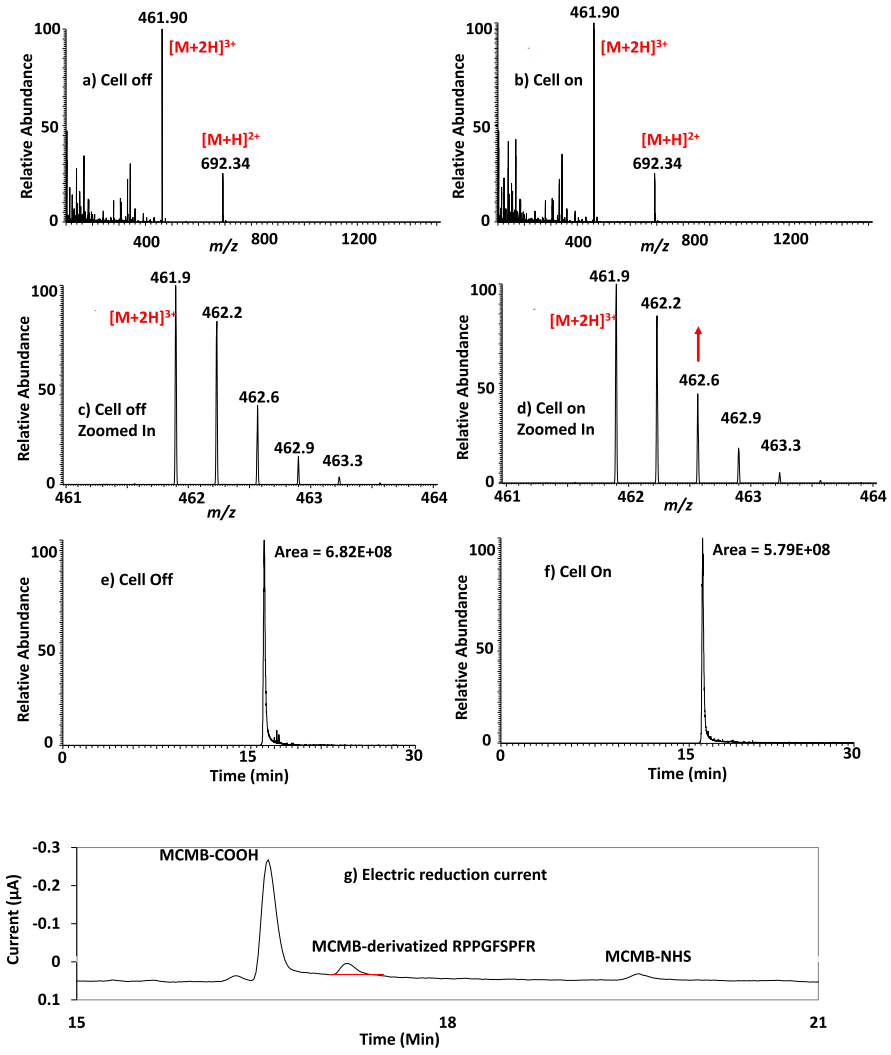

Fig. 2. Mass spectra of MCMB-derivatized RPPGFSPFR a) before electrochemical reduction and b) after electrochemical reduction; Zoomed-in mass spectra of MCMB-derivatized RPPGFSPFR c) before electrochemical reduction and d) after electrochemical reduction; EIC peak of MCMB-derivatized RPPGFSPFR e) before reduction and f) after reduction. g) Diagram showing electrochemical reduction currents.

interest [45–54]. The combination of EC with MS, EC/MS, can be applied to produce drug *in-vivo* metabolites, or cleave proteins/peptides followed with MS analysis [57,58]. It can also be used to reduce disulfide bond to facilitate MS sequencing of proteins/peptides [60–62], and oxidize lipid to determine double bond locations of unsaturated lipids [63,64]. It has also been used to capture elusive reaction intermediates [65–80] and to screen electrosynthetic reactions [81,82]. In our previous work, we have shown that CMS can be used to quantify peptides containing electroactive residues such as tyrosine, tryptophan or cysteine [33,35,36]. However, it is limited to the peptides containing oxidizable residues. In consideration of the idea that peptide can be tagged with an electroactive group such as methylene blue, our goal in this study is to develop an absolute quantitation CMS method for peptides that are not electroactive. The reagent we chose for peptide derivatization in this study was MCMB-NHS ester. The methylene blue moiety of MCMB-NHS ester can be electrochemically reduced (a two-electron reduction process, Scheme 1). The other end of the reagent has a NHS group which is widely used to react with either *N*-terminal or lysine amine group of peptides.

3.1. MG peptide quantitation

MG peptide was first chosen as a test peptide. Through the experimentation we understood that there are two important criteria to be met to enable effective NHS reaction with peptides. The first criteria is the pH control. pH 8 is the most appropriate condition for the derivatization reaction. Using buffers to maintain the pH 8 required desalting before mass spectrometric analysis to prevent mass spectrometric signal loss and instrument contamination. However, we observed sample loss during the desalting procedure, so we used water (pH was adjusted to 8 by NaOH) as the solvent for the derivatization reaction. The second key point is to control the concentration of the derivatizing reagent (MCMB-NHS ester) Vs. the peptide or protein. A high concentration of the derivatizing reagent could ensure the peptide derivatization in a high yield. In this case, a 20 fold MCMB-NHS ester in relative to the MG was used for peptide derivatization. After derivatization, 97.2 % of the peptide was reacted (EIC peak area before derivatization: 6.22E7; EIC after derivatization: 1.78E6).

For the CMS quantitation, 10 μL of 1.5 μM derivatized MG peptide solution was injected for CMS quantitation (total amount of the peptide injected: 15.0 pmol) and a small potential of −0.2 V (vs. Ag/AgCl) was applied to the working electrode of the flow cell for peptide reduction.

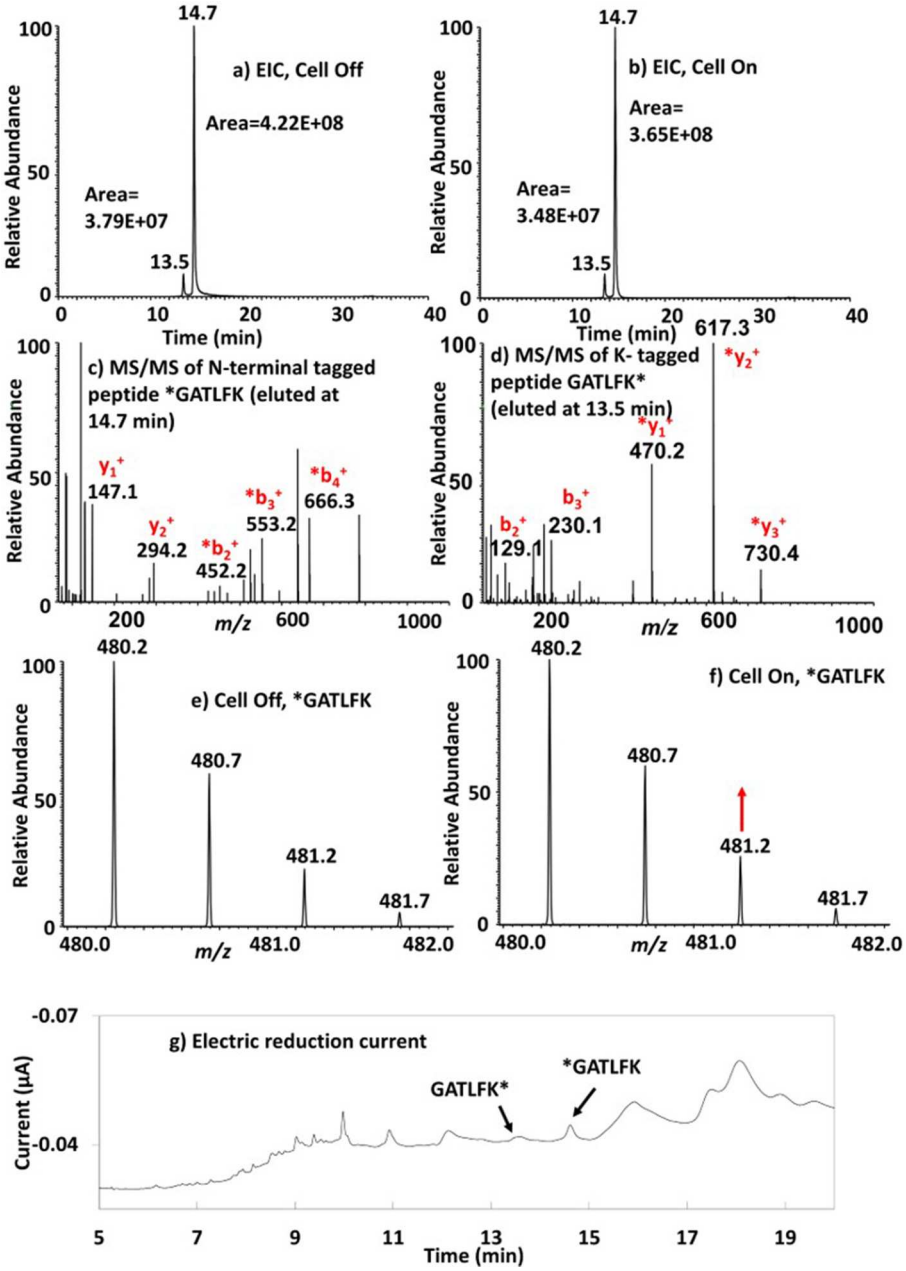

Fig. 3. EIC peaks of MCMB-derivatized peptide GATLFK from cytochrome C a) before and b) after electrochemical reduction; two derivatized peptide peaks are found; MS/MS spectra of c) *N*-terminal derivatized *GATLFK eluted at 14.7 min and d) K-derivatized GATLFK* eluted at 13.5 min; * marks on the tag of MCMB; Zoomed-in mass spectra of *N*-terminal derivatized *GATLFK e) before and f) after electrochemical reduction; g) Diagram showing the electrochemical reduction current of LC-separated protein digest.

Before reduction, +1 ion of MCMB-derivatized MG peak was observed at *m/z* 530.19 (Fig. 1a; note that methylene blue carries one positive charge and therefore peptide appears to be +1 without the need of protonation). Upon reduction, the intensity of +1 ion of MCMB-derivatized MG was reduced. On the other hand, a new peak at *m/z* 266.61 emerged (Fig. 1b), corresponding to +2 of the reduced MCMB-derivatized MG. The EIC peak area of MCMB-derivatized MG at *m/z* 530.19 was smaller by 14.1 % after reduction (Fig. 1d), in comparison with its EIC peak before reduction (Fig. 1c), indicating that the reduction yield for the compound was 14.1 %. (Table S1, Supporting Information). Note that, besides using the relative change of the peptide EIC peak area upon electrolysis for calculating the electrochemical reduction yield, another possible way to estimate the electrochemical conversion yield is based on the comparison of ion intensity between the peptide product and the remaining peptide [32].

On the other hand, the total charge involved in the peptide reduction was found to be 3.9E-07C based on the integration of the peptide reduction current with time (Fig. 1e). In Fig. 1e, besides the reduction current peak of MCMB-derivatized peptide MG, the reduction peaks of the remaining MCMB-NHS and its hydrolysis product MCMB-COOH were also observed. The total charge Q along with the reduction yield of 14.1 % was used to calculate the quantity of the derivatized peptide. Based on the Faraday's law ($n = Q/(zF\Delta i)$ and z = 2 in the case of methylene blue reduction, it was found that the measured amount by CMS was 14.8 pmol. Since, only 97.2 % of the peptide was derivatized, the quantity of the peptide measured was 15.2 pmol. In a triplicate measurement, the averaged value of the 3 runs was found to be 15.7 pmol (CV: 11 %, Table S1, Supporting Information). The theoretical

amount of peptide injected for CMS quantification was 15.0 pmol. The quantitation error of this peptide was +4.8 % (Table S1, Supporting Information).

3.2. RPPGFSPFR peptide quantitation

RPPGFSPFR peptide was chosen as another test sample. In this case, a 20-fold of the derivatizing reagent MCMB-NHS ester relative to the RPPGFSPFR was used. The derivatization yield of this peptide was found to be 99.4 %, by comparing the EIC peak area of peptide signal before and after derivatization reaction (EIC peak area before derivatization: 1.68E8; EIC after derivatization: 1.00E6). The derivatized peptide was subject to LC/EC/MS analysis for CMS quantitation. Before reduction, +3 ion of MCMB-derivatized RPPGFSPFR was detected at *m/z* 461.9 (Fig. 2a). When a potential of −0.2 V (vs. Ag/AgCl) was applied for peptide reduction, *m/z* 461.9 intensity dropped. The integrated EIC peak area for *m/z* 461.9 was smaller by 15.1 % after reduction (Fig. 2f), in comparison with that of the peak before reduction (Fig. 2e), indicating that the peptide reduction yield was 15.1 %. In contrast, ion intensity of *m/z* 462.6 corresponding to the reduced peptide product increased from 2.09E6 to 2.26E6 upon reduction (see zoomed-in peaks in Fig. 2c and d).

The total charge Q involved in the reduction in this case was found to be 3.2E-07C (Fig. 2g). The Q value along with the reduction yield was used to calculate the quantity of the derivatized peptide. Based on the Faraday's law ($n = Q/(zF\Delta i)$, it was found that the quantity of the derivatized peptide was 10.9 pmol. Considering the 99.4 % derivatization yield, the total amount of peptide was 10.9 pmol. Again, this value is in excellent agreement with the theoretical peptide amount of 11.3 pmol (in this experiment, 3 μL of 3.75 μM derivatized RPPGFSPFR peptide solution was injected for CMS analysis), with a small quantitation error of −2.7 % (Table S2, Supporting Information).

The sensitivity of CMS in combination with derivatization strategy was also evaluated, using RPPGFSPFR at low concentrations as an example. RPPGFSPFR was reacted with MCMB-NHS ester overnight and the RPPGFSPFR peptide was completely derivatized by MCMB-NHS ester. In our test, a 5 μL of 0.05 μM MCMB-derivatized RPPGFSPFR (injection amount 250 fmol) was injected for CMS quantitation. In a triplicate analysis, the amount of the peptide measured by CMS was 250 fmol, which is in excellent agreement with the theoretical amount of 250 fmol (0 % error, details shown in Table S3, Supporting Information). This result suggests a high quantitation sensitivity and accuracy of our CMS approach (Note that this quantitation limit of 250 fmol refers to the amount of derivatized peptide, the quantitation limit for original peptide could be higher as a low amount of peptide may react slowly in a low conversion yield during derivatization).

3.3. Cytochrome C quantitation

After success in quantitation of peptides that do not have oxidizable residues by CMS in combination with derivatization strategy as shown above, we further applied this method for protein quantitation. The rationale is that protein can be digested into peptides and peptide can be selected and derivatized for CMS quantitation. Cytochrome C (109 amino acids, sequence is shown in SI) was chosen as the first test sample. A 100 μL of 100 μM cytochrome C was digested overnight using 5 μL of 1 μg/μL trypsin. An approximately 1 fold MCMB-NHS ester (0.1 mM) was added to cytochrome C digest (95.2 μM) for derivatization for 1.5 h. The protein:MCMB-NHS ester ratio was optimized to prevent the formation of peptides with multiple tags. Then, a 10 μL of 1.19 μM cytochrome C digest derivatized by MCMB-NHS ester was injected for CMS analysis (the total amount injected: 11.9 pmol) and GATLFK was selected as a surrogate peptide, for the following reasons: a) this peptide was tagged in either *N*-terminal amine (denoted as "*GATLFK") or its K residue (denoted as "GATLFK*") and the peptide with two tags on both *N*-terminal amine and K residue was not observed; thus the sum of *GATLFK and GATLFK* quantities can be used to calculate the amount of GATLFK in the protein digest, in combination with the derivatization yield. An un-derivatized cytochrome C digest sample was run in parallel with the MCMB-derivatized cytochrome C digest sample to calculate the derivatization yield. Based on comparison of EICs of unreacted GATLFK in two digest samples (EIC peak area before derivatization: 1.37E8; EIC peak area after derivatization: 1.05E8), the derivatization yield for GATLFK was found to be 23%. Both *GATLFK and GATLFK* were well separated from other tagged peptides under the LC condition used. To confirm that *GATLFK and GATLFK* were well separated from other tagged peptides, skyline software was used (see the discussion in SI).

In LC/EC/MS analysis for CMS quantitation, before reduction, +2 of MCMB-derivatized GATLFK peptide at *m/z* 480.2 (Fig. 3a) was detected at two retention times (RT) of 13.5 min and 14.7 min, respectively, indicating that one of them was *GATLFK and the other one was GATLFK*. Collision-induced dissociation (CID) MS/MS spectra was performed to confirm their identities. MS/MS spectrum of the major peptide eluting at 14.7 min showed *N*-terminal tagged ions $*b_2^+$, $*b_3^+$, and $*b_4^+$ (* marks the MCMB tag) at *m/z* values 452.2, 553.2, and 666.3, respectively, confirming that this major peptide peak is *GATLFK (Fig. 3c). In contrast, MS/MS spectrum of the minor peptide eluting at 13.5 min displayed fragment ions $*y_1^+$, $*y_2^+$, and $*y_3^+$ at *m/z* 470.2, 617.3 and 730.4, respectively, suggesting that it is GATLFK* (Fig. 3d).

For CMS analysis, upon reduction using a potential of −0.2 V (vs. Ag/AgCl), +2 ion of *N*-terminal-derivatized *GATLFK had reduced intensity and its EIC peak area was smaller by 13.5% after reduction (Fig. 3b), in comparison with that of the peak area before reduction (Fig. 3a), indicating that its reduction yield was 13.5 %. In contrast, intensity of *m/z* 481.2 corresponding to the reduced peptide product increased from 1.25E6 to 1.43E6 upon reduction (see zoomed-in peaks in Fig. 3e and f). The total charge Q involved in the reduction of the *N*-terminal derivatized *GATLFK was found to be 4.54E-08C (Fig. 3g). Based on the Faradays law ($n = Q/(zF\Delta i)$, the quantity of the *N*-terminal derivatized *GATLFK peptide was 1.74 pmol (Table S4, Supporting Information).

Fig. 3g also displayed the reduction current peak of the K residue-derivatized GATLFK*, the minor derivatized peptide product eluting at 13.5 min. Similar to the *N*-terminal-derivatized *GATLFK, the K-tagged GATLFK* was quantified. Upon reduction using a potential of −0.2 V (vs. Ag/AgCl), +2 ion of K-tagged-derivatized GATLFK* had reduced intensity and its EIC peak area was smaller by 8.3% after reduction (Fig. 3b), in comparison with that of the peak area before reduction (Fig. 3a), indicating that its reduction yield for the 3 runs was 8.3 %. The total charge Q involved in the reduction of the K-tagged derivatized GATLFK* was found to be 1.59E-08C (Fig. 3g). Based on the Faradays law ($n = Q/(zF\Delta i)$, the quantity of the K-terminal derivatized *GATLFK peptide was 0.99 pmol. The total amount of the derivatized GATLFK peptide was 2.73 pmol (the sum of *GATLFK and GATLFK*). Considering the 23 % derivatization efficiency, the total amount of the GATLFK peptide in the sample was calculated to be 11.8 pmol. In a triplicate measurement, the average value was found to be 11.6 pmol (Table S4, Supporting Information). The measured amount is in good agreement with the theoretical amount of cytochrome C digest injected (protein amount: 11.90 pmol) with a quantitation error % of −2% (CV of the 3 runs: 2.0 %).

3.4. β-casein protein quantitation

β-Casein (224 amino acids, sequence is shown in SI) was chosen as another protein sample for test. β-Casein protein was digested using trypsin. To 100 μL of 100 μM β-casein, 10 μL of 1 μg/μL trypsin was added for overnight digestion. Then, approximately 8-fold MCMB-NHS ester (0.8 mM) relative to β-casein (90.9 μM) was used for derivatization of the protein digest overnight. 5 μL of 5.05 μM digested β-casein derivatized by MCMB-NHS ester was injected for LC/MS analysis (the theoretical protein amount: 25.3 pmol). A surrogate peptide GPFPIIV was identified and separated for LC/EC/MS analysis. An un-derivatized β-casein digest was run in parallel with the MCMB-derivatized β-casein

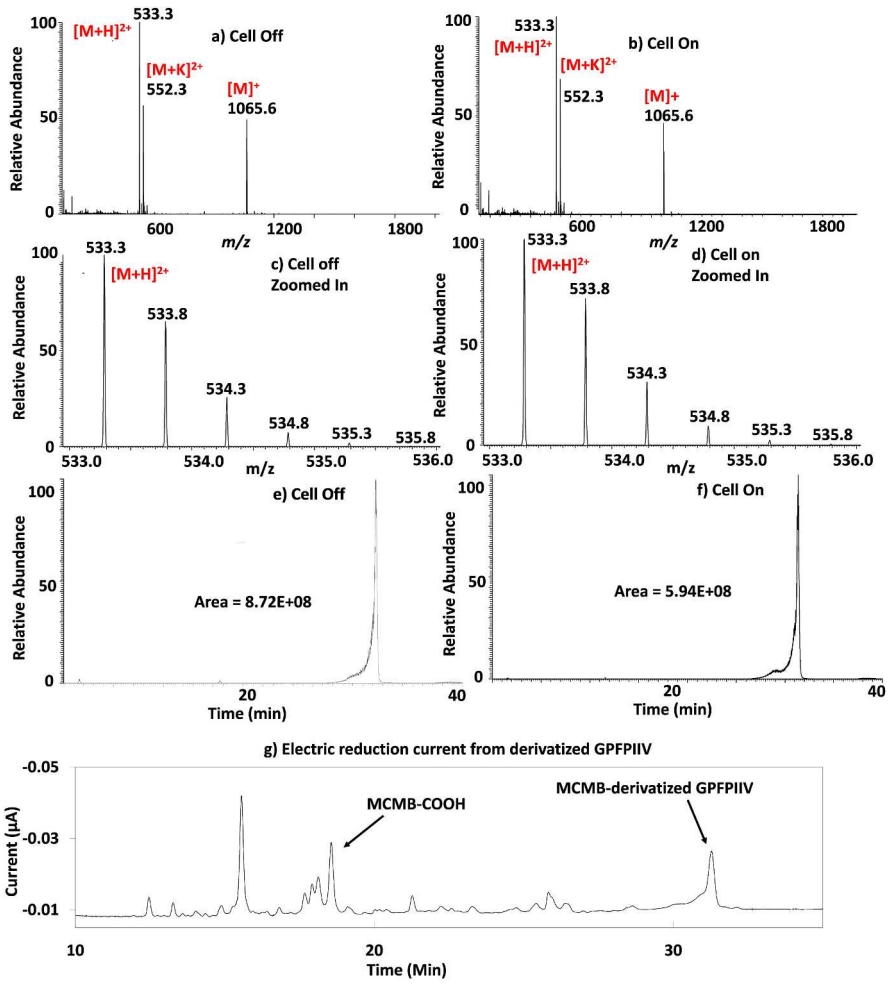

Fig. 4. Mass spectra of MCMB-derivatized GPFPIIV resulting from tryptic digestion of β-casein a) before electrochemical reduction and b) after electrochemical reduction; Zoomed-in mass spectra of MCMB-derivatized GPFPIIV c) before electrochemical reduction and d) after electrochemical reduction; EIC peak of MCMB-derivatized GPFPIIV e) before reduction and f) after reduction; g) Diagram showing the electrochemical reduction currents of LC separated protein digest.

digest to evaluate the derivatization yield. In this case, the EIC peak area of GPFPIIV before reaction was 9.59E7 and decreased to 5.87E7 after derivatization, suggesting that 38.8 % of the GPFPIIV peptide was derivatized with MCMB-NHS ester.

For CMS analysis, +2 of MCMB-derivatized GPFPIIV peptide was detected at *m/z* 533.3 (Fig. 4a). When a potential of −0.2 V (vs. Ag/AgCl) was applied for peptide reduction, the ion intensity of *m/z* 533.3 decreased by 31.9 % (Fig. 4e and F), indicating that the reduction yield for the derivatized peptide was 31.9 %. The total charge Q involved in the reduction was found to be 4.5E-07C (Fig. 4g). Thus, based on the Faradays law ($n = Q/(zF\Delta i)$, the quantity of the derivatized peptide was 7.3 pmol. Since 38.8 % of the GPFPIIV peptide was reacted with MCMB-NHS ester, the total amount of the GPFPIIV peptide in the sample was 7.3/38.8 % = 18.8 pmol. As the theoretical amount of β-casein injected was 25.3 pmol, the quantitation error was −25.9 %. The quantitation result indicates that there might be some sample loss during the process of tryptic digestion of β-casein protein to peptides [83].

4. Conclusions

In this study, electrochemically inactive peptides MG and RPPGFSPFR peptides were successfully quantified using CMS, after their derivatization with MCMB-NHS ester carrying a methylene blue moiety. It provides a general approach to quantify peptides. The method is further applicable to protein quantitation, as demonstrated by CMS analysis of cytochrome C and β-casein proteins. High quantitation sensitivity of 250 fmol (using RPPGFSPFR) was also achieved. The striking strength of this method is that it requires no standard/isotope-labelled peptides for absolute quantification and the method can be generally applied to various peptides/proteins. The small reduction potential (−0.2 V vs. Ag/AgCl) of methylene blue electrochemical tag provides the method selectivity, as other peptides would not be reduced at such a low potential. Overall, the method reported in this study may have a good potential of applications in proteomics research.

Credit author roles

Praneeth Ivan Joel FNU: Methodology, Formal analysis, Investigation, Writing - Original Draft, Visualization, Data curation.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijms.2023.117153.

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